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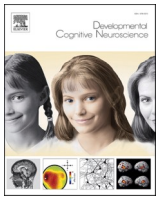

Cortical reorganization during adolescence: What the rat can tell us about the cellular basis

Janice M. Juraska [a,b,*], Carly M. Drzewiecki [a]

[a] Program in Neuroscience, University of Illinois at Urbana-Champaign, Champaign, IL, 61820, United States
[b] Department of Psychology, University of Illinois at Urbana-Champaign, 603 E Daniel St., Champaign, IL, 61820, United States

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ABSTRACT

The human cortex, particularly the prefrontal cortex, decreases in volume during adolescence which indicates considerable pruning. There is consistent evidence from human, monkey and rat tissue that synapses, dendritic spines and dendrites are pruned during this time. However, our work with a rat model of adolescence shows that other cellular components are remodeling at this time as well. Neurons are also pruned and we have found that in female rats, puberty is a key signal for this process. Other critical developmental events occur that are not detectable in gross size changes including the growth of dopaminergic inputs. The changes in the inhibitory GABAergic system, especially the parvalbumin-expressing neuronal subtype, are an essential part of the maturation of the prefrontal cortex. This involves the formation of perineuronal nets around parvalbumin interneurons that allow mature fast spiking. We have found a large increase in perineuronal nets from early adolescence to adulthood in both sexes. We also have seen a temporary pause in this increase at the time of puberty in females. These complicated events cannot be deduced from MRI. The cellular reorganization that is indicated by size changes in the human cortex during adolescence can be informed by work from rodent models.

Since the seminal MRI studies of Giedd et al. (1999) and Sowell et al. (1999), anticipated by Jernigan et al. (1991), it is clear that the structure of the human cerebral cortex changes during adolescence. This work shows a peak in cortical gray matter volume early in adolescence, which is especially prominent in the prefrontal cortex, followed by a decrease. These changes occur in both sexes and may involve the hormones secreted during puberty (Koolschijn et al., 2014; Lenroot and Giedd, 2006).

For a volume change to be detectable at the gross size level in MRI, considerable pruning at the cellular level must occur, though exactly what is pruned is difficult to establish and beyond the scope of current MRI technology. The observations of cellular changes during adolescence in humans and rhesus monkeys have a number of unavoidable limitations, not the least being a small number of subjects. Rodent models of cellular rearrangements in the adolescent cerebral cortex offer more opportunity for understanding the details of cellular changes as well the influences of sex and pubertal hormones. In comparison to primate and human autopsy tissue, the brains of rodents can be examined at selected ages in relatively large numbers, and their short life cycle makes raising them affordable. Fig. 1 illustrates the timeline of adolescence and puberty within the lifespan in humans and rats.

In the present review, we do not attempt to cover all of the rodent literature, nor do we review the array of changes that have been found throughout the human adolescent brain with MRI (e.g., Brown et al., 2012). Rather, we give a brief overview of the cellular findings from the primate literature (including humans), followed by a focus on what has been found in our laboratory in rats that could underlie the loss of volume seen in human adolescents.

1. Cortical changes in humans and rhesus monkeys during adolescence

The studies of cellular changes in humans and monkeys have several themes in common: they focus on the dorsolateral prefrontal cortex (Brodman area 9) in a set of subjects that are predominantly male. These studies also involve a small number of subjects during the adolescent period but they cover a broad range of ages before and after adolescence that tend to bolster their conclusions. The highly cited Huttenlocher

Abbreviations: mPFC, medial prefrontal cortex; E, embryonic day; P, postnatal day; PL, prelimbic region of the medial prefrontal cortex; IL, infralimbic region of the medial prefrontal cortex; VO, ventral orbital cortex; VLO, ventrolateral orbital cortex.

* Corresponding author at: Department of Psychology, University of Illinois, 603 E Daniel St., Champaign, IL, 61820, United States.
E-mail address: jjuraska@illinois.edu (J.M. Juraska).

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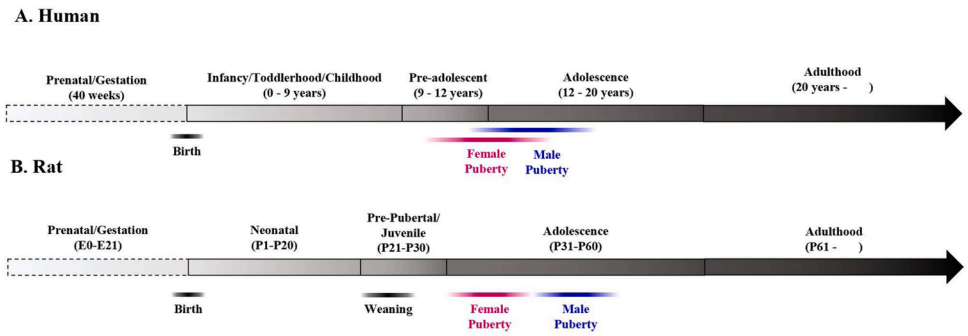

Fig. 1. Developmental timelines including adolescence and puberty of humans (A) and rats (B). It should be noted that the timeline for rats does not hold necessarily for mice since inbred strains often differ. (E = embryonic day; P = postnatal day).

(1979) study actually only examined one (!) adolescent but the observation that synapses are lost during adolescence in humans has been sustained by subsequent studies. Petanjek et al. (2011) found dendritic spine density in Golgi stained layer III pyramidal neurons peaks before adolescence as assessed in three adolescent subjects. Since the majority of synapses in the cerebral cortex are found on dendritic spines, this indicates that synapse density decreases during adolescence. Glantz et al. (2007) had the largest group of adolescent subjects with nine from ages 11–15 years and four from 16 to 20 years. The study examined the synaptic marker, synaptophysin, in Western blots subjects and showed there was a significant decrease from 6 to 10 years to 16–20 years. All of these studies support the idea that synapses peak before or early in human adolescence and then are later lost.

Examination of rhesus monkeys indicate that prefrontal synapses are pruned during adolescence as well. Bourgeois et al. (1994) quantified prefrontal synapses with electron microscopy in five adolescents, and their graphs of synaptic density indicate a peak early in the juvenile period followed by a decrease towards puberty and another decrease during adolescence. The authors also attempted to compensate for differences in the thickness of the cortex by calculating synaptic density within a single dorsal to ventral probe which at least partially represents the volume differences that occur in three-dimensions. No statistics were performed so that conclusions come from observation of the graphs. However, Anderson et al. (1995) did statistically support similar conclusions. Looking at layer III pyramidal neurons, they report prefrontal spines decrease between pre- and post-pubertal animals on both apical and basilar dendrites. Notwithstanding a low number of subjects (3/age group) and some of the males having been castrated, the decrease was statistically significant. They also examined the dendritic length of the apical portion of the dendritic tree and found no statistical differences across ages. However, there was a numerical decrease in the post-pubertal age group which indicates greater decreases in the total number of spines than simple density (number/dendritic length) indicates.

In spite of the inherent limitations of the studies in human and monkey tissue, they are concordant in finding that spines and synapses decrease across adolescence. They leave many open questions, however, including whether there are sex differences and if the hormones secreted at puberty are playing a role. Puberty is a long process in human adolescents and its status in tissue donors is not known, leaving a role for animal studies in which large subject numbers and simple assessment of puberty is possible. Also, one can question whether the magnitude of the decline in synaptic density, along with concomitant dendritic/axonal loss, is sufficient to be readily detectable as a volume decrease in structural MRI.

2. The rat as a model

Based on the defining input from the mediodorsal thalamic nucleus, the rat has two major prefrontal cortical regions: the ventromedial, which is subdivided into prelimbic (PL) and infralimbic (IL), and the orbitofrontal cortex, composed of ventral orbital (VO) and ventrolateral orbital (VLO) (Uylings et al., 2003). Although both prefrontal regions have executive functions, in our work we have concentrated on PL and IL which we will refer to as the medial prefrontal cortex (mPFC). We also use the Long-Evans hooded rat which is outbred and has pigmented eyes

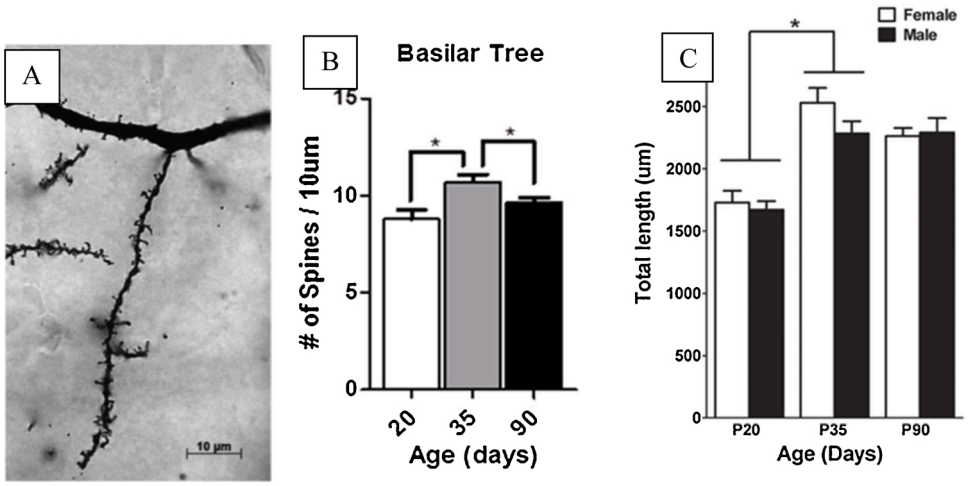

Fig. 2. The dendritic tree of layer V pyramidal neurons in the mPFC. A. A picture of dendritic spines on an apical branch visualized with Golgi-Cox. B. The density of dendritic spines peaks in adolescence at P35 in both sexes. C. The length of the basilar dendritic tree grew from P20 to P35 but did not significantly decrease by P90, although Sholl analysis showed decreases in portions of the tree in females. $p < .05$ (Koss et al., 2014).

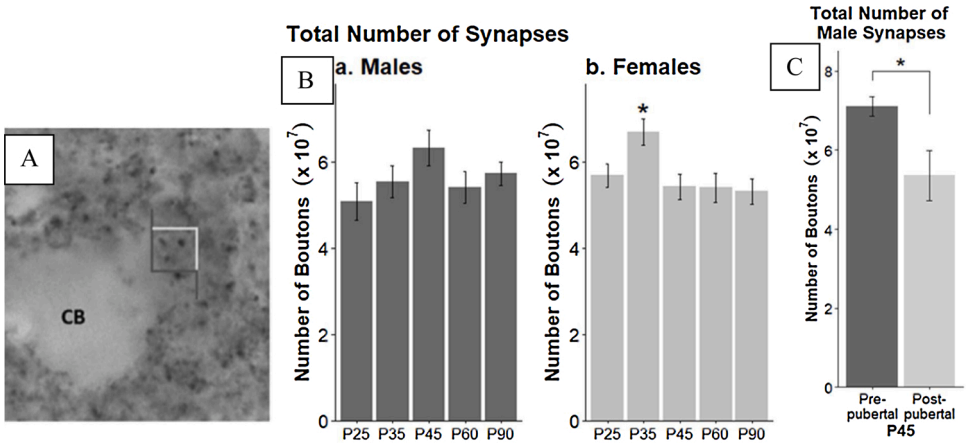

Fig. 3. Synaptophysin labelled synapses. A. A picture of the synaptophysin boutons with an optical disector counting frame in the mPFC. CB is cell body. B. The number of synapses peaked at P35 and was higher than both P25 and P45 in females. No overall significant differences were found for males. C. However, on the average day of male puberty (P45), pre-pubertal had more boutons than post-pubertal males. $p < .05$ (Drzewiecki et al., 2016).

which enable more acute vision than the red eyes of albinos. This is an advantage in behavioral tasks but will not be reviewed here.

Puberty has been shown to be a relatively abrupt and externally visible event in rats of both sexes. There is a correlation between rising testosterone levels and preputial separation in male rats (Korenbrot et al., 1977) making pubertal status readily assessable on a particular day. Likewise, female rats exhibit vaginal opening that correlates with a rise in estrogen and luteinizing hormone surges that are essential components of the estrous cycle (Castellano et al., 2011). This allows for the detection of the day of pubertal onset in rats. This is not readily done in female mice where the rise in estrogen and beginning of the estrous cycle are not tightly correlated (Safranski et al., 1993; Clark and Price, 1981). We have consistently found that female rats reach puberty 7–10 days earlier than male rats. This is important because knowing the day when hormones rise can help establish the role of puberty in the rapid changes occurring during adolescence.

3. Pruning of dendrites, dendritic spines and synapses

Like the data from primates including humans, we have found that there is pruning of dendrites and dendritic spines in the mPFC of rats. Quantifying Golgi-Cox impregnated pyramidal neurons in layer V of the mPFC (Fig. 1A) (N = 5–7/sex/group), we found that spine density decreased on the basilar dendritic tree between adolescence (postnatal day (P) 35) and adulthood (P90) in rats of both sexes (Koss et al., 2014) (Fig. 2B). This result is consistent with the pruning reported in primates, including humans. However, there were no overall decreases in dendritic branching between adolescence and adulthood at least in this cell population (Fig. 2C). Nonetheless, there were indications from a Sholl ring analysis that females did have decreases in dendritic length between 60−100 μm from the cell body. The total number of spines on a neuron is calculated as spine density (number/length) multiplied by dendritic length. Groups can have equal spine density but if one has a larger dendritic tree, it has more total spines. Therefore, females are pruning more spines and therefore, more excitatory synapses, than males. However, by representing adolescence with P35, we realized that the male peak at puberty onset during adolescence may have been missed.

This motivated us to quantify synapses using synaptophysin examining more ages across the adolescent period. Synaptophysin is a glycoprotein in synaptic vesicles and thus is a marker for synapses which allows for individual synapses to be immunolabelled and manually counted (Fig. 3A). Again, this density count was multiplied by the volume of the mPFC for each animal resulting in the total number of synapses. Five ages were quantified spanning the juvenile period to adulthood with 9–11 animals/age/sex. From daily assessment of the markers of puberty, the average age of puberty was P35 for females and P45 for males. Fig. 3B indicates that the number of synapses increased from P25 with a peak on the day of puberty in both sexes (Drzewiecki et al., 2016). The peak was significant in females with the number of synaptophysin boutons at P35 being significantly larger than either P25 or P45 (Fig. 3B). Although the overall age effect was not significant in males, a comparison of males that had reached puberty by P45 with those that had not at this age showed that there was a decrease in the number of synapses following puberty (Fig. 3C).

Puberty appears to be an important modulator of synaptic pruning in both sexes. Whether it is necessary in the long term will require further experiments with pre-pubertal removal of ovaries and testes and examining whether age may slowly accomplish the pruning that gonadal hormones induce quickly. Boivin et al. (2018) found that ovarian hormones did not influence the ultimate density of spines on the upper apical dendrites in the mouse cingulate cortex, an area adjacent to the mPFC. It is unclear if this is a regional/species difference in the role of ovarian steroids, or simply due to the compensating effects of age or an indication that the density does not represent total spine number since changes in the length of the dendritic tree are not known.

The results from Drzewiecki et al. (2016) indicate that the lack of pruning of the dendritic tree in males between adolescence and adulthood in Koss et al. (2014) may have been due to the day chosen to represent adolescence. This day, P35, was compared to adulthood when the males were prepubertal and continuing to grow their dendritic tree while females were at or near puberty when pruning would predominate. The non-overlapping ages at which male and female rats reach puberty means that age and pubertal status are readily dissociable but that no single age can adequately represent adolescence.

It should be noted that a decrease in the number of synapses actually represents a complex combination of synaptic loss and gain with loss being numerically larger. This has been demonstrated in the mouse in an area adjacent to the mPFC, the dorsal anterior cingulate, with two-photon microscopy (Johnson et al., 2016). This supports earlier work indicating continued growth of amygdalar axons into the mPFC during late adolescence (Cunningham et al., 2002). The rearrangements within the prefrontal cortex notwithstanding, there is an overall loss of prefrontal grey matter that is large enough to be consistently detected in MRI. Our studies in rats are concordant with the work in humans and rhesus monkeys that part of the volumetric loss in the adolescent to adult transition involves pruning of synapses. Furthermore, our work has added evidence of a modulatory role for puberty.

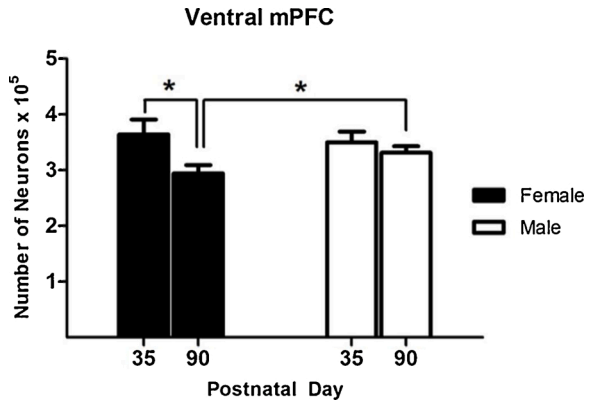

Fig. 4. The loss of neurons between adolescence and adulthood in the rat prefrontal cortex. There was a significant loss in both sexes that occurred predominantly in females between P35 and P90. This resulted in a sex difference in adulthood. $p < .03$ (Markham et al., 2007).

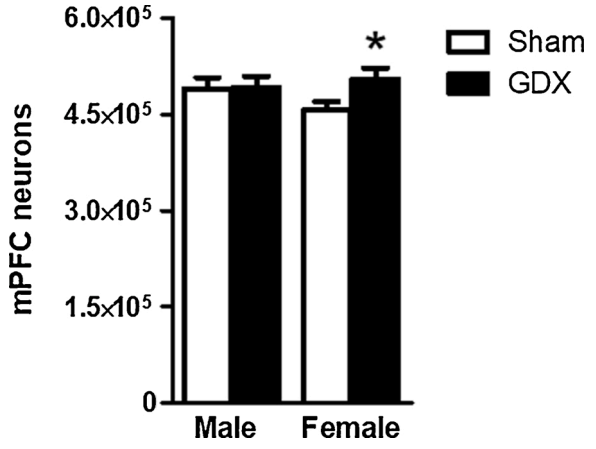

Fig. 5. The number of adult neurons in the mPFC in rats in which the gonads (testes and ovaries) were removed before puberty. $p < .05$ (Koss et al., 2015).

4. Pruning of neurons

Like the investigators using MRI or examining human tissue, we did not hypothesize that whole neurons were part of the pruning processes during adolescence. We encountered this possibility during an investigation of the origin of sex differences in the number of neurons in the adult rat visual cortex. We found that prepubertal removal of the ovaries resulted in more neurons in the female primary visual cortex that was indistinguishable from the number in males, while removal of the testes in prepubertal males was without effect (Nuñez et al., 2002). Since postnatal neurogenesis in the cortex is near to zero (Ehninger and Kempermann, 2003), including during adolescence (Koss and Juraska, unpublished data), there must be apoptosis during adolescence.

In the rat mPFC, we established that the number of neurons decreases in both sexes between adolescence and adulthood (P35 and P90) (Markham et al., 2007) but the degree of loss was considerably larger in females (Fig. 4). This was confirmed in a subsequent study (Willing and Juraska, 2015) with a larger set of ages in which females had a significant decrease in the number of neurons between ages P35 (puberty) and P45. Further support comes from a study in which we found that prepubertal gonadectomy (removal of the testes in males and ovaries in females) at P20 resulted in a higher number neurons in females without ovaries compared to in intact females. In contrast, the number of neurons in the adult male mPFC was unaffected by the prepubertal removal of testes (Koss et al., 2015) (Fig. 5). All of these studies imply that the ovarian steroids increase apoptosis.

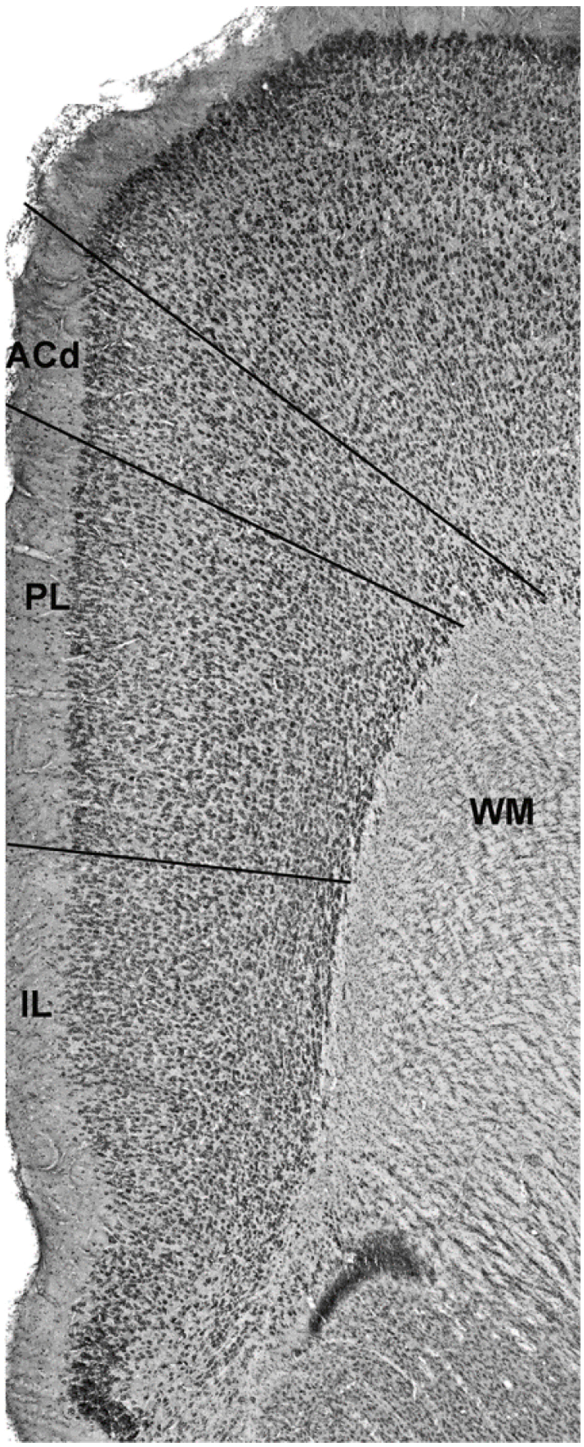

Fig. 6. A coronal view of the rat mPFC stained with methylene blue/azure II, a Nissl stain. PL and IL are portions of the mPFC. ACd is the dorsal anterior cingulate and WM is the white matter. (Markham et al., 2007) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

A sizable decrease in the number of neurons during adolescence would be a major contributing factor to the detectability of the volume loss of grey matter during human adolescence. The studies reported above all examined from 9 to 11 rats/sex/age in coded tissue so that the phenomenon of adolescent apoptosis is not a statistical fluke. Yet, the possibility of neuron loss during adolescence has not been considered in the literature involving humans nor has it been further investigated in

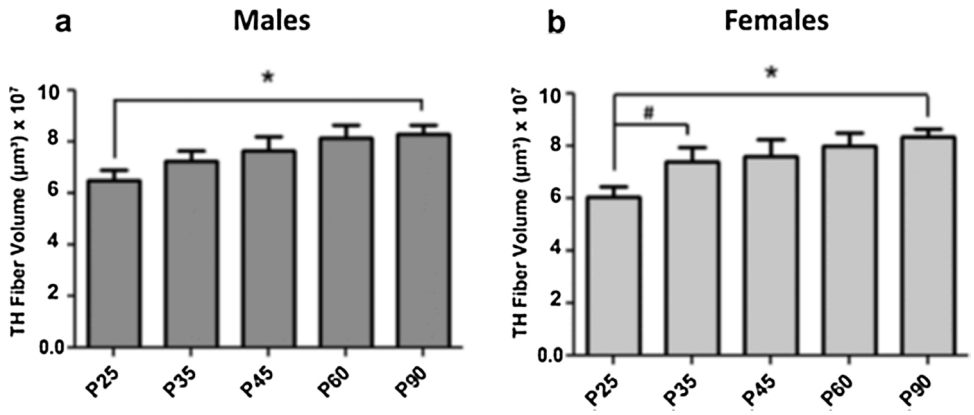

Fig. 7. The total volume of the tyrosine hydroxylase containing axons in the rat mPFC in males (a) and female (b). *p < .05 (Willing et al., 2017).

other mammalian species. This may be because it seems a radical process for an already functioning nervous system. Indeed, many investigators who do not study early cellular neurodevelopment know little about apoptosis in general; whereas synapse formation and loss can occur through the lifespan and are widely studied and acknowledged.

Another reason that neuronal loss is ignored is that it is time consuming to investigate. Simple neuronal density (number/volume) only indicates the distance between cells and is dependent on the composition of the neuropil. Density from counts using the optical dissector must be multiplied by the volume of the neural region under study to know the total number of anything. This requires parcellation of the cortical areas which can be accomplished across sections using a simple Nissl stain because there are subtle difference in lamination and packing density between cytoarchitechtonic areas in the mPFC as illustrated in Fig. 6. Parcellation is theoretically possible in human autopsy tissue but would be especially difficult given the variability of human tissue and the sulci/gyri that would make following cytoarchitechtonic characteristics difficult to define.

5. Changes not detectable with MRI

5.1. Dopamine

In addition to neuronal and synaptic losses, there is continued growth of several transmitter systems into the mPFC as alluded to earlier. Among them is the growing dopaminergic system (Kalsbeek et al., 1988; Hoops et al., 2018). We quantified the growth of axons carrying tyrosine hydroxylase (the rate limiting step for dopamine synthesis) across adolescence with regard to both sexes and puberty. A definitive role for puberty was not apparent (Willing et al., 2017) (Fig. 7).

5.2. Perineuronal nets

Remodeling of the inhibitory GABAergic system, especially the fast spiking neurons that express parvalbumin (PV), is an important part of adolescent development of the mPFC (reviewed in Caballero and Tseng, 2016). The formation of perineuronal nets (PNNs) around PV neurons contribute to their maturation. PNNs are specialized components of the extracellular matrix found throughout the central nervous system (Celio et al., 1998; Seeger et al., 1994). PNNs in the mPFC primarily surround PV cells, where they act as a cation buffer to enhance the excitability of the fast-spiking interneurons (Balmer, 2016; Härtig et al., 1999). PNNs also contribute synaptic stability and create a barrier that hinders the formation of new synaptic contacts while stabilizing existing ones (Härtig et al., 1992).

The maturation of PNNs had been shown to contribute to the end of the critical period for ocular dominance in the mouse visual cortex

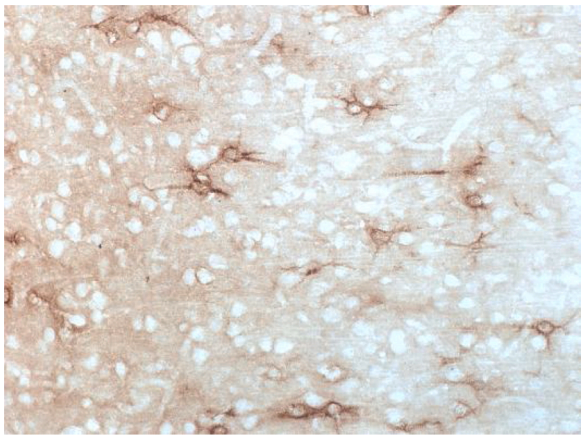

Fig. 8. PNNs, surrounding the cell bodies and proximal dendrites, were visualized with *Wisteria floribunda* in rat mPFC.

(Pizzorusso et al., 2002) and it has been hypothesized to contribute to the closure of critical periods in humans (reviewed in Reh et al., 2020). PNNs have been found in the human prefrontal cortex and they increase in density across adolescence (Mauney et al., 2013), and Baker et al. (2017) found that PNN density increases between adolescence and adulthood in male rats. All of the above studies examined only male subjects, with the exception of Mauney et al. that had such a small number of females that no differences could be discerned.

We have recent, as yet unpublished, work quantifying PNN numbers in female rats with an emphasis on puberty in both sexes. We visualized PNNs with *Wisteria floribunda* agglutin (Fig. 8), and we have quantified their number (density multiplied by mPFC volume) across adolescence. Both sexes showed large increases from prepuberty (P30) to young adulthood (P60) as previously described in male rats (Baker et al., 2017). To explore the role of puberty, we compared sibling females in which one had reached puberty and the other had not and found that there was an abrupt and significant decrease in the number of PNNs in post pubertal females (Drzewiecki, Willing and Juraska, unpublished data; submitted). This decrease persisted for at least a week, eventually rebounding to adult-like levels by P60. The number of PNNs in males was not directly impacted by pubertal onset. The implications for the temporary pause in maturation of the inhibitory system in postpubertal females is not yet known, but if a similar phenomenon occurs in the human prefrontal cortex, it would not be detectable with MRI.

5.3. Corpus callosal axons

The last example of cellular remodeling that is not detectable in gross

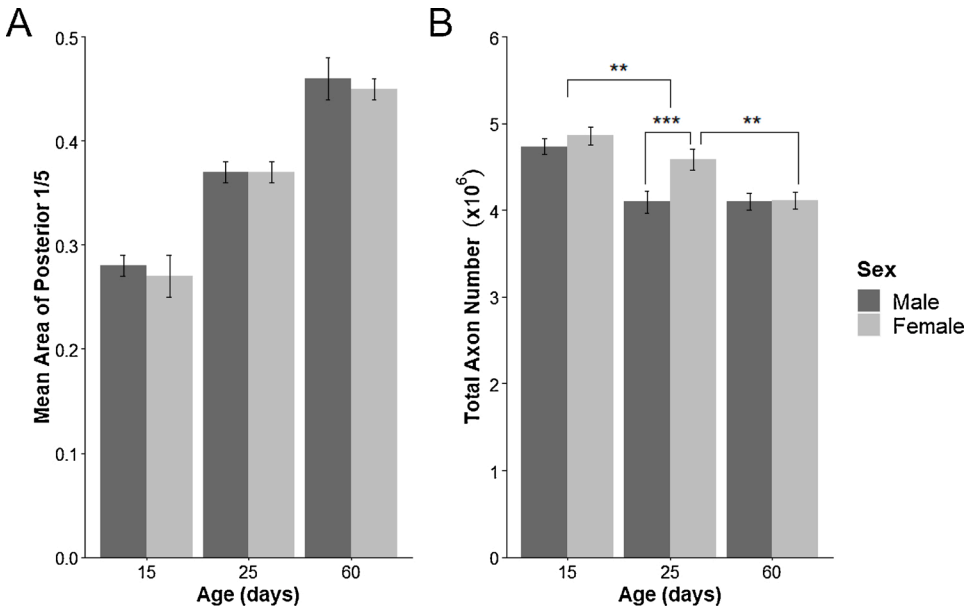

Fig. 9. The size (A) and number of axons (B) of the splenium (posterior 1/5) of the corpus callosum. All of the ages were significantly different than each other ($p < .0001$) in A. **$p < .005$; ***$p < .0005$. (Kim and Juraska, 1997).

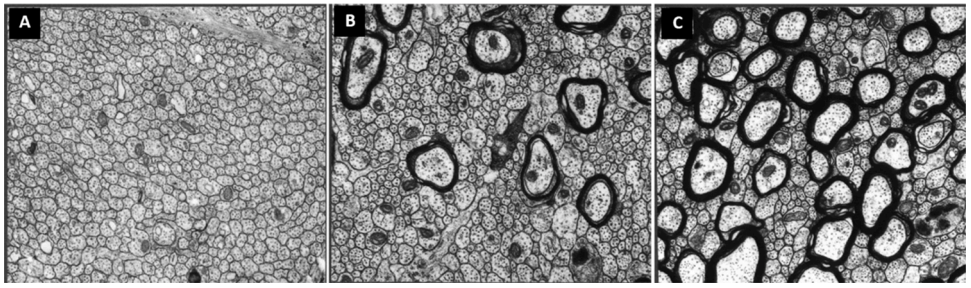

Fig. 10. Electron micrograph in from the splenium of the rat corpus callosum at different ages. The darkly stained circular structures are myelin. A. P15; B. P25; C. P60. (Kim and Juraska, 1997).

size changes is from our work in the posterior region (splenium) of the corpus callosum. This study occurred before we were attending to adolescence as an important time period and before the decrease in cortical volume in human adolescents was widely known. We found that the midsagittal area of the splenium increases from P15 to P25 to P60 in rats of both sexes with no sex differences at these ages (Fig. 9A). In spite of the large increase in area (50 %) across these ages, a constant proportion (posterior 1/5) of the whole structure carries the axons from the visual cortex. We thoroughly sampled the posterior 1/5 with electron microscopy and found obvious increases in the number of myelinated

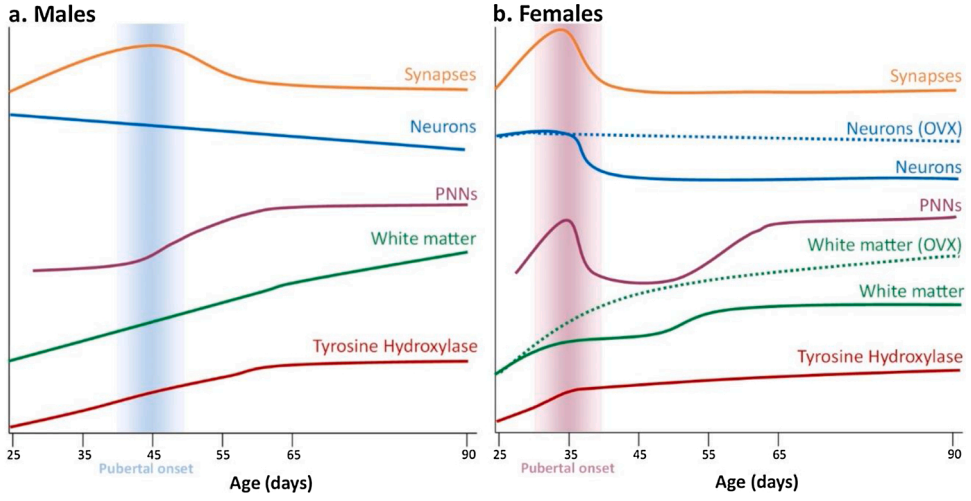

Fig. 11. A representation of the cellular measures discussed above in the rat mPFC.

axons between each age that accounted for the expanding area of the structure (Fig. 10) (Kim and Juraska, 1997). What was unexpected, however, was that even while the size of the splenium was increasing, axons were being pruned in both sexes but at different times (Fig. 9B). In a later study, we found that the axon loss was not altered by prepubertal ovariectomy, so that this is a process not influenced by female puberty (Yates and Juraska, 2008). This is another illustration that the cellular components underlying differences in the size of a structure cannot be deduced from gross size changes. Additionally, puberty does not inevitably influence every cellular change. Here, there is pruning in the midst of increasing size that is masked by other cellular processes.

6. Conclusions

Adolescence is a dynamic period of neural remodeling in the cortex, some of which is reflected in changes in volume that can be measured with MRI images in humans. There is consistent evidence of pruning of synapses, and concurrently spines and dendrites, across mammalian species. Data from rats indicates that neurons may also be pruned in the human cortex, but this will be difficult to demonstrate with current methodologies.

Given that the hormones secreted at puberty play an important role in the remodeling occurring during adolescence in rodents, and this is especially pronounced in females, pubertal status should be noted whenever possible. Evidence implies a role for pubertal onset in human cortical maturation as well (e.g. Lenroot & Geidd, 2006; Herting and Sowell, 2017), though we are cognizant that disentangling age-related changes from hormonally-driven maturation will be a difficult task.

More coordination between the literature on non-human animals and humans is called for, although there are probably species differences in terms of timing if not of type of changes. However, it is important to note that not all cellular elements change in the same direction during adolescence (Fig. 11), and many of the additions in terms of new input and other modifications like perineuronal nets are not detectable in the size of the cortex.

CRediT authorship contribution statement

Janice M. Juraska: Conceptualization, Writing - original draft. **Carly M. Drzewiecki:** Writing - review & editing, Visualization.

Declaration of Competing Interest

The authors report no declarations of interest.

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